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PRINCIPAL INVESTIGATOR: Xiaoxing Wang, Ph.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02215

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14. ABSTRACT I hypothesize that metastasis is governed by specific genetic alterations and that identifying this genetic program will provide candidates for new therapeutic targets. My aim is to discover potential metastasis suppressor genes (MSGs) in murine model of breast cancer metastasis through genome scale loss-of-function studies. As a first step to establish a system for pre-screening candidate MSGs among the vast number of gene candidates, I calibrated two in vitro tumor invasion assays, metastasis initiation assay and Matrigel invasion assay. Especially, the Matrigel transwell assay has superior robustness that allow me to further develop assay in both arrayed and pooled format. In parallel, I have pursued an in vivo strategy to identify metastasis-related genes using an in vivo cell type-specific lentiviral gene delivery system. I first confirmed that this gene delivery system efficiently permits gene expression in an in vitro cell culture model. Next, I tested the lentiviral gene delivery system in an in vivo subcutaneous tumor model. Virus was introduced through tail vein injection offers reasonable virus delivery efficiency. I am currently testing this in a murine model of tumor formation.					
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Introduction

Work from many laboratories has identified the function of several oncogenes and tumor suppressor genes in their role of cancer initiation. However, we continue to lack insight into the molecular program that leads to metastasis. I hypothesize that metastasis is governed by specific genetic alterations and that identifying this genetic program will provide candidates for new therapeutic targets. My aim is to discover potential metastasis suppressor genes (MSGs) in murine model of breast cancer metastasis. To systematically screen for MSGs, I will use genome-scale lentiviral-based RNAi reagents that permit large scale loss-of-function studies. I will then focus on candidate MSGs that are altered in human metastatic breast cancer by integrating data from high-density single nucleotide polymorphism arrays and expression profiling of human breast cancer cell lines and patient specimens. Selected putative MSGs will be investigated for their role in metastasis in a series *in vitro* and *in vivo* metastasis assays.

Body

The focus and accomplishment at this stage of work include the following two aspects:

Aim 1: To evaluate *in vitro* assays that model metastasis for screening candidate MSGs

Recent genomic, transcriptomic and proteomic analysis of human breast cancer reveal large number of genes that correlate with breast cancer metastasis (Ref 1-5). In order to select a list of MSG candidate genes for the *in vivo* MSGs characterization, I attempted to develop a robust *in vitro* metastasis assay that allows me to screen a large panel (in order of couple hundred) of candidate MSGs.

Metastasis is a complex process composed of multiple steps, and prior work suggested that some of these steps can be modeled by *in vitro* assays that delineate specific stages of this disease, such as enhanced motility, invasion and angiogenesis. One such assay previously developed by S. Gobeil et al (Ref 6) using mouse melanoma cells has the advantage to characterize both cell invasion and motility in a three-dimensional cell culture system. In this system, tumor cells are embedded in collagen which is coated with Matrigel and sandwiched between two layers of fibrin gel. Tumor cells that have invasive and mobile ability are able to migrate from collagen gel into fibrin gel and form individual colonies. Both migrating and non-mobile tumor cells can be isolated for further characterization and continuous culture. This assay is compatible with a pooled screening format.

To adapt this assay, I chose positive control cell lines: MDA-MB-231 and PC3, both of which form metastasis in immunodeficient mice when introduced through tail vein or orthotopic injection. First, I optimized the pH condition for cell to proliferate in collagen gel as appropriate pH is required for both collagen gel polymerization and cell proliferation. Cells (5×10^4) were mixed with collagen gel solution (1.5mg/ml final concentration) in 200ul volume in one well of a 96-well plate. To titrate pH, various amount of sodium bicarbonate solution was added to the mixture to promote collagen polymerization. To monitor cell proliferation, cells were recovered from collagen gel and cell viability was measured by CellTiter-Glo (Promega). Cell proliferation was monitored for four days and the optimal pH for each cell type was determined (Figure 1).

Then these cells were assayed for colony formation by embedding the cell-collagen plug in fibrin gel to form collagen gel-fibrin gel sandwich. The growth of cells was monitored for up to six weeks under light microscope. Unlike previous report that metastatic cells migrated from collagen gel and formed satellite colony in fibrin gel, both MDA-MB-231 and PC3 cells migrated into fibrin gel forming a continuous network (Figure 2 a and b). I concluded that this assay does not permit the isolation of individual colonies with distinct invasive/motile characteristics. Therefore, this assay could not be adapted as an approach to pre-screen MSGs.

Another *in vitro* assay I tried to modify for the screen is Matrigel transwell assay (BD Biosciences) which measures the mobility of cells and their ability to degrade extracellular matrix (ECM). Two metastatic cell lines (MDA-MB-231 and PC3) and one non-metastatic cell line (MCF7) were seeded at various densities in the assay chamber with or without Matrigel cover to assay cell invasion or motility, respectively. Forty-eight hour post cell seeding, cells were fixed and nuclei was stained with DAPI. In a parallel experiment, cells were recovered from assay chamber and cell viability was measured by CellTiter-Glo. The two metastatic cell lines displayed higher invasive ability and motility comparing to the non-metastatic MCF7 cell line, with MDA-MB-231 scoring significantly higher on both attributes (Figure 3 a and b). This assay appears to have high signal-to-noise ratio. I plan to test the pro-invasive effect of putative MSGs on non-metastasis cell lines using this assay. We currently have five shRNAs designed against various regions of putative MSG genes, including ARHGDIB, CDH1, CRSP3, DAB2IP, DNAJB4, EZH2, GAS1, HOXD10, PEBP1, PRDM13 and SETD2.

Aim 2: To develop an *in vivo* breast cancer model for genome scale loss of function screen

In parallel to Aim 1, I am establishing an *in vivo* screening strategy to identify MSGs. All current *in vivo* screening strategies involve infecting cells *in vitro* and subsequently transplanting the infected cells into nude mice. The disadvantages of this approach are: (1) Cells used in the screen may not be the tumor cells of origin. Cancer cell lines as a primary source for screening have experienced extensive genetic alterations during *in vitro* cell culture and *in vivo* tumor progression. Therefore, their genetic context and physiological behavior deviate from condition in which tumor grows; (2) For most cancer cell lines, only a fraction of cells can initiate tumor after transplant *in vivo*. Therefore the efficiency of using this system to screen a large panel of candidate is compromised; (3) To screen human cancer cell lines in immunocompromised mice cannot account the effect of host immune system towards tumor formation and metastasis. To overcome these problems, I decide to test an established virus infection system which could potentially enhance *in vivo* infection efficiency. This system takes advantage of avian sarcoma-leukosis virus (ASLV) which specifically recognizes and infects cells with retroviral TVA receptor. Transgenic mice with TVA receptor under the control of mouse mammary tumor virus (MMTV) promoter are currently available. Somatic delivery of either mouse polyoma virus middle T antigen (PyMT) or neu oncogene using ALSV-A-based retroviral vector to MMTV-TVA mice induced the formation of induced multiple, oligoclonal tumors within 3 weeks in infected mammary glands that displayed greater cellular heterogeneity than did tumors arising in MMTV-PyMT mice (Ref 7). To improve gene delivery, B. Lewis et al developed a human immunodeficiency virus type 1 (HIV-1)-based lentiviral vector, pseudotyped with the envelope protein of ALSV subgroup A (EnvA) (Ref 8). The ASLV as a virus delivery system will allow us to study tumor formation and metastasis with mammary origin in non-immunocompromised mice *in vivo*.

To test the feasibility of this system, I first compared the infection efficiency of ALSV (EnvA pseudotyped) to the vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped lentivirus vectors. Three tumor cell lines (MCF7, A549 and PC3) were infected with EnvA or VSV-G pseudotyped lentivirus expressing shRNA against TBK1 or EGFR. The depletion of protein was detected by Western blot at three and four days after virus infection. The suppression of target genes by shRNA against TBK1 or EGFR was comparable using lentivirus pseudotyped with either envelope protein (Figure 4).

Next, I tested the infection efficiency of EnvA lentivirus in tumor *in vivo*. A549 cancer cell line expressing TVA and yellow fluorescence protein (YFP) were injected to nude mice subcutaneously and formed palpable tumor after 2 weeks. Lentiviral expression vector which carries genes coding red fluorescence protein (RFP) was injected through either tail vein or directly into the tumor twice per week for 6 weeks. Tumor was then harvested and processed for FACS analysis. Virus introduced directly into the tumor failed infection. Infection efficiency of virus through tail vein injection achieved 5-25%

(Figure 5). No apparent correlation between tumor size and infection efficiency was observed. Further experiments is planned to titrate virus dosage and administration frequency to achieve better infection efficiency.

Key Research Accomplishments

- Evaluated two *in vitro* metastasis assays as pre-screen tools for MSGs discovery *in vivo*
- Assessed an *in vivo* virus delivery system for infection efficiency

Reportable Outcomes

Not Available

Conclusion

The aim of my research is to identify MSGs in a murine model of metastatic breast cancer. As a first step to establish a system for pre-screening candidate MSGs among the vast number of gene candidates, I calibrated two *in vitro* tumor invasion assays, metastasis initiation assay and Matrigel invasion assay. After a series of optimization experiments, I determined that the metastasis initiation assay could not give robust phenotypic readout for high throughput screening. Alternatively, the Matrigel transwell assay allowed me to distinguish metastatic and non-metastatic cell lines in terms of mobility and invasiveness. In parallel, I have pursued an *in vivo* strategy to identify metastasis-related genes using an *in vivo* cell type-specific lentiviral gene delivery system. I first confirmed that this gene delivery system efficiently permits gene expression in an *in vitro* cell culture model. Next, I tested the lentiviral gene delivery system in an *in vivo* subcutaneous tumor model. Virus was introduced through two methods: tail vein injection and direct injection into tumor. I found that the tail vein injection method offers higher virus delivery efficiency. I am currently testing this in a murine model of tumor formation.

References

1. Ding L, Ellis MJ, Li S, Larson DE, Chen K et al. 2010. Genome remodelling in a basal-like breast cancer metastasis and xenograft *Nature* 464:999-1005
2. Shah SP, Morin RD, Khattra J, Prentice L, Pugh T et al. 2009. Mutational evolution in a lobular breast tumour profiled at single nucleotide resolution *Nature* 461:809-13
3. Ellsworth RE, Seebach J, Field LA, Heckman C, Kane J et al. 2009. A gene expression signature that defines breast cancer metastases *Clin. Exp. Metastasis* 26:205-13
4. He M, Mangiameli DP, Kachala S, Hunter K, Gillespie J et al. 2010. Expression signature developed from a complex series of mouse models accurately predicts human breast cancer survival *Clin. Cancer Res.* 16:249-59
5. Ho J, Kong JW, Choong LY, Loh MC, Toy W et al. 2009. Novel breast cancer metastasis-associated proteins *J. Proteome. Res.* 8:583-94
6. Gobeil S, Zhu X, Doillon CJ, Green MR. 2008. A genome-wide shRNA screen identifies GAS1 as a novel melanoma metastasis suppressor gene *Genes Dev.* 22:2932-40

7. Du Z, Podsypanina K, Huang S, McGrath A, Toneff MJ et al. 2006. Introduction of oncogenes into mammary glands in vivo with an avian retroviral vector initiates and promotes carcinogenesis in mouse models *Proc. Natl. Acad. Sci. U. S. A* 103:17396-401
8. Lewis BC, Chinnasamy N, Morgan RA, Varmus HE. 2001. Development of an avian leukosis-sarcoma virus subgroup A pseudotyped lentiviral vector *J. Virol.* 75:9339-44

Appendices

Not Available

Supporting Data

Figure 1. Cell Proliferation in Collagen Gel under Different pH Conditions

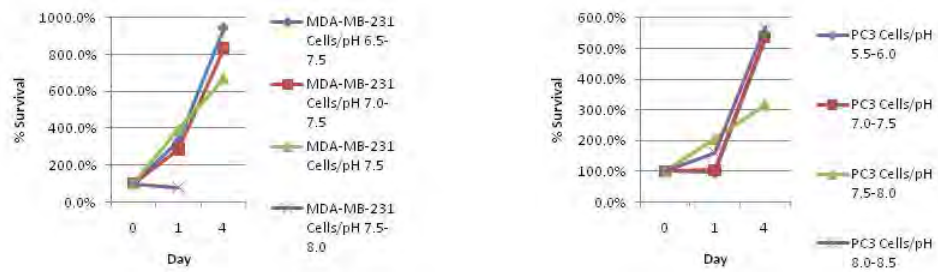


Figure 2. a MDA-MB-231 Cells Colony Formation Efficiency

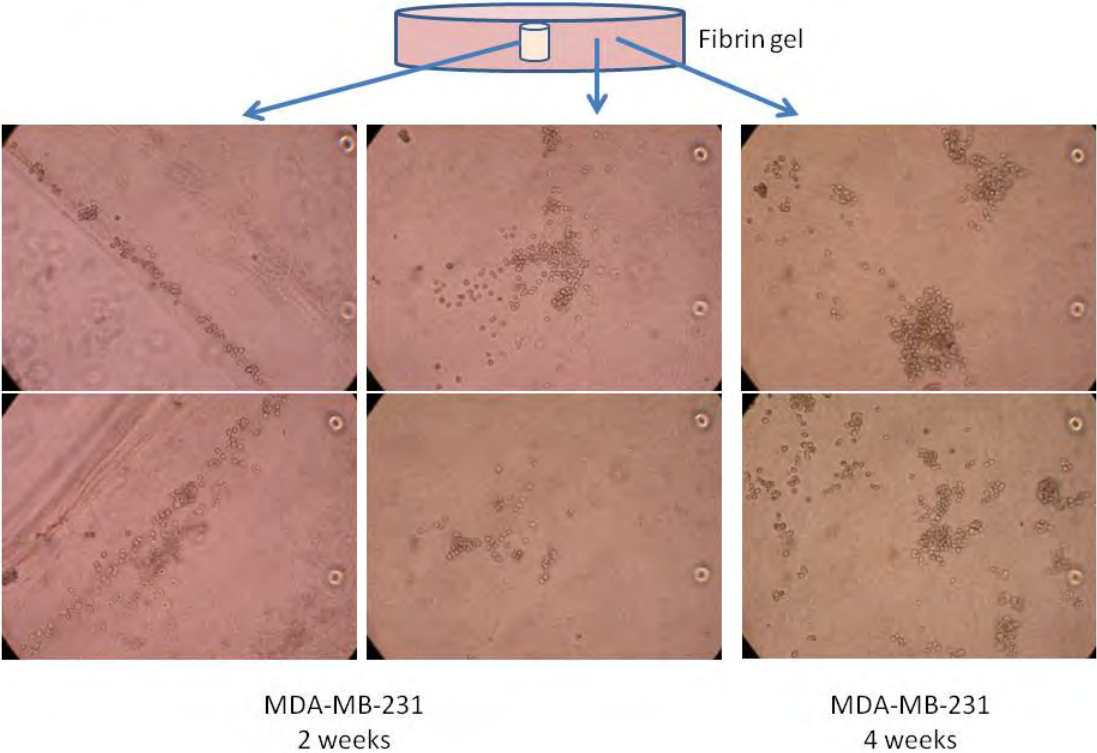


Figure 2. b PC3 Cells Colony Formation Efficiency

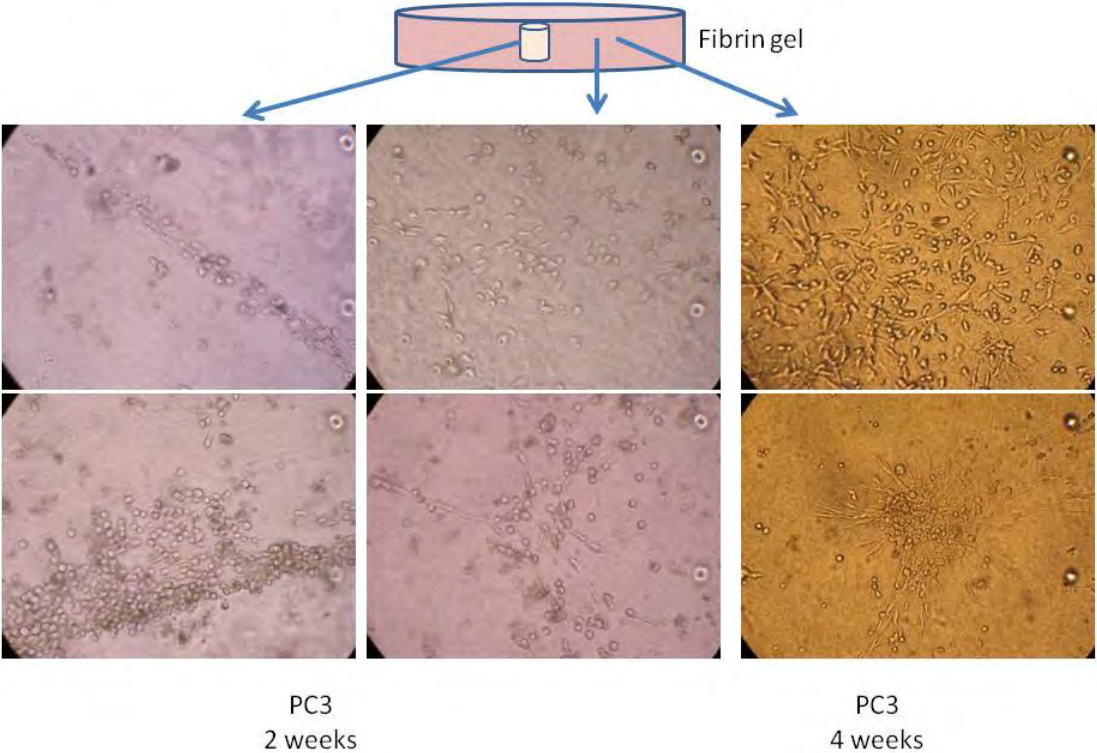


Figure 3. a BD Matrigel Invasion Assay-Motility

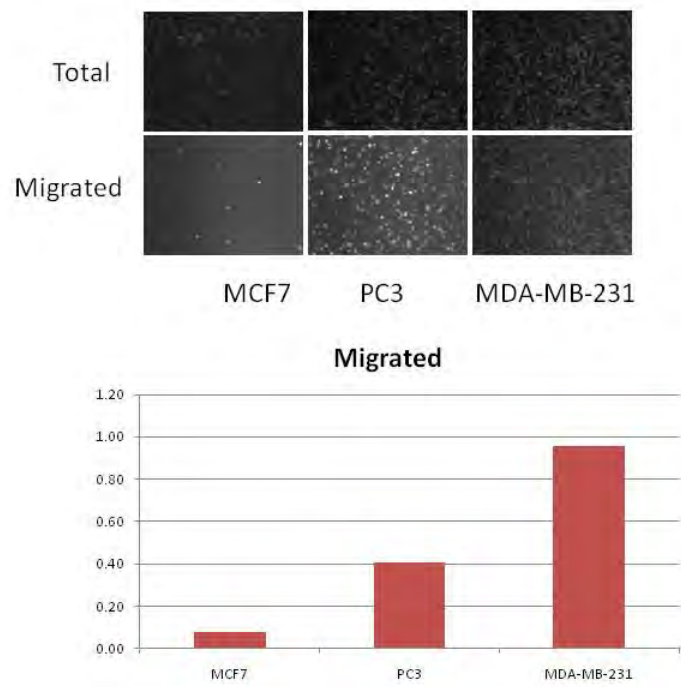


Figure 3 b BD Matrigel Invasion Assay-Invasion

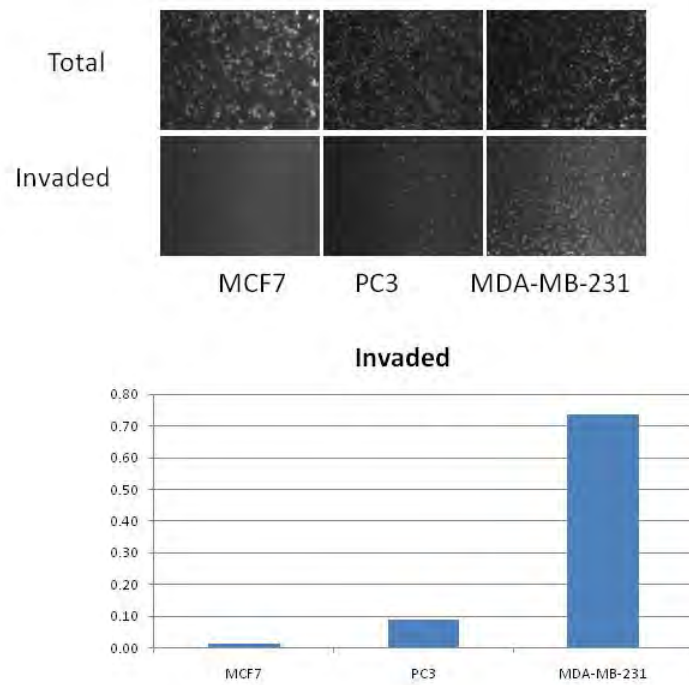


Figure 4. Gene Delivery Efficiency is Comparable for EnvA and VSVG Lentiviruses

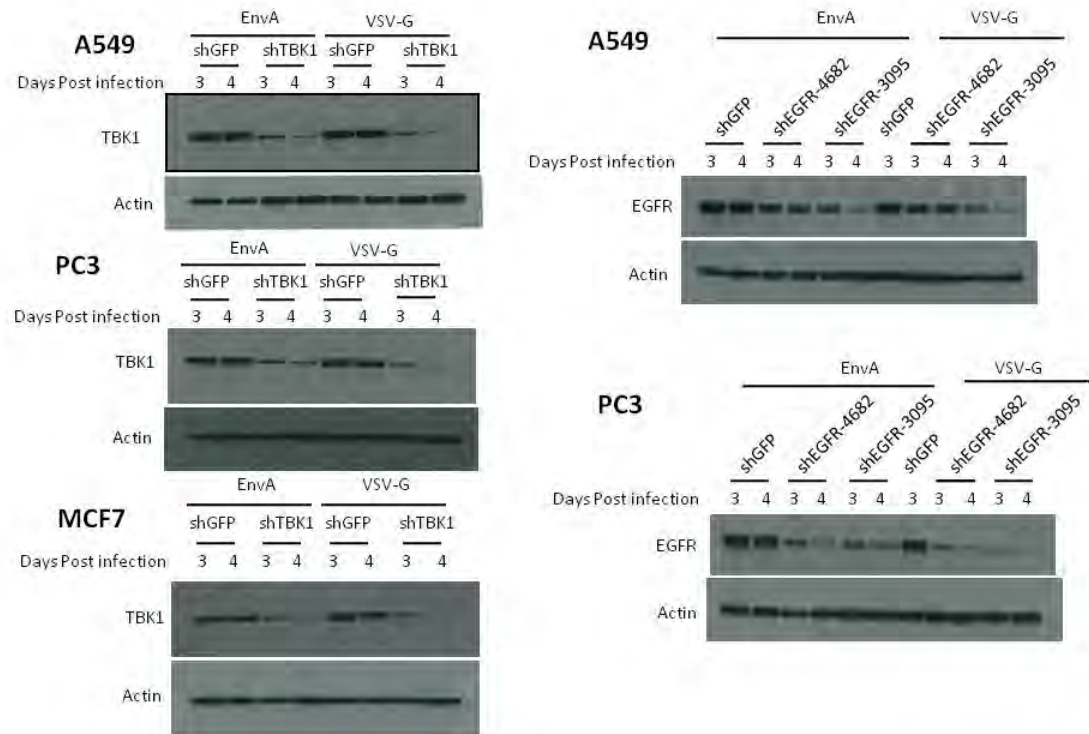


Figure 5. EnvA Lentivirus Infection *in vivo*

